Barrier Function of Cultivated Limbal and Oral Mucosal Epithelial Cell Sheets

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PURPOSE. To investigate barrier function of cultivated limbal and oral mucosal epithelial sheets.

METHODS. Human and rabbit cultivated limbal epithelial sheets (CLES) and cultivated oral mucosal epithelial sheets (COMES) were subjected to immunohistochemistry for keratin K3, K4, K13, ZO-1, and occludin. Results were compared with those obtained from normal cornea and oral mucosa. Prepared under various culture conditions, the in vitro barrier function of these sheets was evaluated by biotin assay and transepithelial electrical resistance measurement. The CLES and COMES were transplanted to rabbit eyes with limbal deficiency, and fluorophotometry was performed before and after surgery. Transplanted corneas were subjected to immunohistochemistry after surgery.

RESULTS. Both normal corneal epithelium and CLES were positive for K3 but negative for K13. Both CLES and COMES showed positive expression of ZO-1 and occludin in the apical epithelium. Both CLES and COMES demonstrated tight junction-like structures in the apical portion of the superficial epithelium by electron microscopy. Although rabbit COMES showed better barrier function than CLES, as evaluated by biotin assay and transepithelial electrical resistance, no such differences were noted in human sheets. Whereas epithelial barrier function was significantly improved after transplantation of CLES in the rabbit limbal deficiency models, recovery was not remarkable after COMES transplantation. Immunohistochemical study revealed that transplanted COMES had an irregular epithelial thickness with decreased expression of ZO-1 in some areas.

CONCLUSIONS. Epithelial barrier function was influenced by various factors such as culture condition and local environment after transplantation. CLES appears to offer better barrier function than COMES. (Invest Ophthalmol Vis Sci. 2009;50: 5672–5680) DOI:10.1167/iovs.09-3820

One of the major functions of the ocular surface epithelia is that of a barrier. Impaired epithelial barrier function often results in invasion by pathologic organisms, with subsequent tissue destruction and scarring. In corneal epithelium, tight junctions (TJs) are well formed and are responsible for its excellent barrier properties.1 TJs consist of multiple transmembrane proteins, scaffolding, and signaling proteins. TJ transmembrane proteins include occludin, claudin, and junctional adhesion molecules. In addition, zona occludens proteins such as ZO-1, ZO-2, and ZO-3 interact with transmembrane proteins at the cytoplasmic face of the cell membrane, serving to anchor them to the actin cytoskeleton. Occludin, claudin, and ZO-1 are expressed in superficial epithelial cells of the cornea.2

The development of new surgical methods making use of ex vivo cultivated epithelial cell sheets has brought in a new era in ocular surface reconstruction. A number of reports have shown encouraging short-term clinical results since the first report by Pellegrini in 1997,3–18 However, the long-term efficacy and safety of this approach remains to be confirmed. In particular, only a few studies have investigated the functional properties of the cell sheets themselves. Although expression of TJ-related proteins in cultivated epithelial sheets has been reported, their function remains to be studied.19,20 We found that transplanted epithelium was remarkably permeable to sodium fluorescein when measured using fluorophotometry in patients who had undergone cultivated oral mucosal epithelial transplantation (COMET).21 Another report indicated that transplanted oral mucosal epithelium was easily distinguishable by slit-lamp biomicroscopy because it stained well with fluorescein dye.7 These reports suggest that oral mucosal sheets have decreased barrier function compared with normal corneal epithelium. However, it remains to be determined whether this decrease in barrier function results in susceptibility to invasion of the corneal stroma by pathogenic organisms.

The purpose of the present study was to investigate barrier function in cultivated epithelial sheets. Expression of TJ-related proteins and cytokeratins was examined by immunohistochemistry. ZO-1 and occludin were studied because they were extensively examined not only in the corneal epithelium but also in other types of stratified epithelia, including oral mucosal epithelium.1 Expression of cytokeratins, including K3, K4, and K13, were also studied using immunohistochemistry. K3 is known as a marker of the corneal epithelium, though it was shown to be expressed in normal and cultivated oral mucosal epithelium. K4 and K13 are expressed in nonkeratinized epithelia, including oral mucosal and conjunctival epithelium, but not in the corneal epithelium.22,23 Their expression pattern has been used for the characterization of cultivated epithelial sheets.24–25 Epithelial barrier function was studied by electrical resistance measurement and dye tracing methods. In vivo barrier function was also studied in both limbal and oral mucosal epithelial sheets using animal experimental models.

METHODS

Antibodies

Mouse monoclonal antibodies for cytokeratin K3, K4, K13, occludin, and ZO-1 were purchased from Progen (A85; Heidelberg, Germany), DBS (6810; Pleasanton, CA), YLEM (Ks13.1; Rome, Italy), Invitrogen (OC-3F10: Carlsbad, CA), and Invitrogen (Z01–1A12), respectively. Rhodamine- and Cy3-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA) and Chemicon International Inc. (Temecula, CA).

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Preparation of Cultivated Epithelial Sheets

All experimental procedures and protocols were approved by the Animal Care and Use Committee of Tokyo Dental College and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell Source

Both human and rabbit tissues were used as donor cell sources for producing epithelial sheets. Normal human and rabbit corneas and oral mucosa were used as controls. Human corneas were obtained from eye banks in the United States for investigational purposes. Limbal rims of corneoscleral tissue were prepared by careful removal of excess sclera, iris, and corneal endothelium. Epithelial sheets were isolated as described previously.6 Human oral mucosal tissues were obtained from patients undergoing COMET. After sterilizing the oral cavity, inferior buccal mucosa was excised using an 8-mm-diameter biopsy punch (KAI Industries Co., Ltd., Gifu, Japan) under local anesthesia. Rabbit limbal and oral mucosal tissues were prepared from female Japanese white rabbits (Japan CLEA, Tokyo, Japan) weighing 2.5 to 3.0 kg each. Anesthesia was introduced by intravenous injection of 4 mL pentobarbital sodium (50 mg/mL). Mucosal specimens were dissected, and submucosal connective tissue was removed with scissors. The epithelium was cut into small pieces and washed several times to remove blood and adipose tissue in Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 mixture at a ratio of 1:1 (vol/vol; Invitrogen Corporation, Grand Island, NY) with 10% fetal bovine serum (FBS), 5 g/mL gentamicin (Invitrogen), and 0.25 μg/mL amphotericin B (Sigma, St. Louis, MO).

Preparation of Cell Sheet

Cultivated limbal epithelial cell sheets (CLES) and oral mucosal epithelial cell sheets (COMES) were used in the present study. Both types of cell sheet were prepared with and without underlying substrates. Preserved human amniotic membranes (AMs) were used as substrates. Both epithelial cell sheets with and epithelial cell sheets without substrates were studied to examine whether the presence of AM influences barrier function. Different methods for cell preparation for cultivation, cell suspension, and explant methods were used to study whether the culture methods affected the results.28 AMs were donated by mothers who were seronegative for human immunodeficiency virus and hepatitis B and C virus at the time of cesarean section. Written informed consent was obtained from donors, and the procedure was performed in accordance with the Declaration of Helsinki. AMs were stored in 15% dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO) with PBS at 80°C until use. Denuded AM was prepared as described previously.28 Membranes were rinsed in PBS, spread onto the upper chambers of culture inserts (Transwell, cat. no. 3450; Costar Corning, Corning, NY), frozen at −80°C, and air dried at room temperature.

Preparation of Substrate-Free Cell Sheets

For preparation of substrate-free cell sheets, fibrin sealant (Bolheal; Fujisawa Pharmaceutical Co., Osaka, Japan) was constituted as reported previously.27 In brief, a solution containing 40 mg human fibrinogen and 0.18 U thrombin was diluted with 7.5 mL saline, and 0.5 mL resultant solution was spread rapidly onto each of the upper chambers of the culture inserts (Transwell; Costar Corning). Two hours later, the polymerized fibrin-coated upper chambers were retrieved and stored at 4°C.

Cell Suspension Culture

The basal cells of the limbal and oral mucosal epithelium were harvested after enzymatic treatment (Dispase II; Roche Diagnostics, Indianapolis, IN) at 37°C for 1 hour, followed by 0.05% trypsin-0.53 mM EDTA solution (Invitrogen) at room temperature for 10 minutes. Cell suspension was washed in DMEM/F12 medium (1:1 mixture) with 10% FBS. Cell suspension was resuspended with supplemental hormonal epithelial medium (SHEM) containing 666 KIU/mL aprotinin (Wako, Osaka, Japan).16,27 It was then seeded (1.0–2.0 × 105 cells/well) onto fibrin or human-denuded AM on the bottom of the culture plate inserts (Transwell; Costar Corning) in a 6-well plate (Corning) containing mitomycin-C (Sigma) treated with 3T3 fibroblasts (2.0 × 105 cells/cm²). The culture was submersed in medium for 7 to 10 days and exposed to air by lowering the level of the medium at the end of the culture period.

Explant Culture

After careful removal of the excess sclera, iris, corneal endothelium, and conjunctiva, corneoscleral tissues were cut into approximately 2 × 2-mm squares. Two pieces with the epithelium side up were directly placed onto the fibrin- or AM-coated insert and were covered with SHEM for 2 days. The culture was submersed in medium until it reached confluence, after which it was exposed to air at the end of the culture period.

Transmission Electron Microscopy

Human CLES and COMES were fixed in 2.5% glutaraldehyde solution in 60 mM HEPES buffer solution for 4 hours. After washing, samples were postfixed in 1% osmium tetroxide, dehydrated in a series of ethyl alcohol and propylene oxide, and embedded in epoxy resin. Semithin sections (1 μm) were stained with toluidine blue. Ultrathin specimens were then sectioned with a microtome (LKB, Gaithersburg, MD). Sections in the range of gray to silver were collected on a 150-mesh grid, stained with uranyl acetate and lead citrate, and examined under an electron microscope (model 1200 EXII; JEOL, Tokyo, Japan).

Histology and Immunohistochemistry

CLES and COMES cultivated on AMs were subjected to immunohistochemistry for the detection of ZO-1 and occludin, and the results were compared with those from normal human cornea and oral mucosa. Frozen sections were fixed for 10 minutes in cold acetone (occludin, ZO-1; Wako) or 2% paraformaldehyde (K3, K13; Wako). Sections were blocked by incubation with 10% normal donkey serum (Chemicon International Inc.) and 1% bovine serum albumin (Sigma-Aldrich) for 1 hour at room temperature. Antibodies to occludin (1:50), ZO-1 (1:50), K3 (1:1000), and K13 (1:30) were applied and incubated for 90 minutes at room temperature, followed by incubation with rhodamine- or Cy3-conjugated secondary antibody. After three washes with TBST, the sections were incubated with 1 μg/mL 4,6-diamidino-2-phenyldole (DAPI; Dojindo Laboratories, Tokyo, Japan) at room temperature for 5 minutes. Finally, the sections were washed three times in TBST and coverslipped after mounting with an antifade medium (50 mM Tris buffer saline, 90% glycerin; Wako), and 10% 1,4-diazabicyclo-2,2,2-octane (Wako).

Assessment of Epithelial Barrier Function Using Biotin Assay

Epithelial barrier function was evaluated using an assay (LC-Biotin; Pierce, Rockford, IL) that has been reported to cross-link to proteins and that does not penetrate intact tight junctions.26,28 First, the culture medium was gently aspirated, and the cell sheets were irrigated twice with SHEM supplemented with 5% FCS. Assay (1 mg/mL in PBS; LC-Biotin; Pierce) was applied to the apical side of the cell sheets for 1 minute, followed by removal of the medium and two washes with PBS. Penetration of the assay was determined by immunofluorescence assay using fluorescein-conjugated streptavidin antibody (Jackson ImmunoResearch). Grade of dye penetration was semiquantitatively assessed as follows: grade 0, no penetration; grade 1, penetration as far as superficial layer of epithelium; grade 2, penetration throughout epithelium. Three different areas were examined, and grading was averaged in each sample.

Transepithelial Electron Resistance Measurement

Transepithelial electrical resistance (TER) measurement was performed in the CLES and COMES using the epithelial volt ohm-meter (World Precision

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Instruments, Sarasota, FL). Cultures were equilibrated at room temperature for 10 to 15 minutes before electrical measurement. During measurement, the apical and basal chambers were filled with 1 mL and 2 mL medium, respectively. Resistance was expressed in ohms.

Epithelial Sheet Transplantation to Rabbits
Rabbit CLES and COMES were transplanted to rabbit corneas with limbal deficiency. All animals were handled in full accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and institutional guidelines. Rabbits were anesthetized with intramuscular injection of xylazine hydrochloride (2.5 mg/mL) and ketamine hydrochloride (37.5 mg/mL). The left eye in each rabbit was rendered totally limbal stem cell deficient. An 8.5-mm superficial incision was made using the Hessberg-Barron trephine (Katena Products, Denville, NJ). Superficial keratectomy of the peripheral cornea involving the limbus and neighboring bulbar conjunctiva, followed by mechanical debridement of the central corneal epithelium, was performed using a keratectomy knife (Grieshaber, Microsurgery Knife 681–25; Aton, Switzerland). Autologous limbal epithelium from the superficial keratectomy or oral tissue excised using an 8-mm diameter biopsy punch (KAI Industries Co., Gifu, Japan) was used for epithelial sheet cultivation. After application of antibiotic ointment (0.3% ofloxacin; Tarivid; Santen Pharmaceutical Co., Osaka, Japan), the corneas were examined twice a week.

Two weeks after keratectomy, epithelial sheet transplantation was performed in eyes with limbal deficiency. After scraping off all fibrovascular tissue from the cornea and neighboring bulbar conjunctiva, either autologous rabbit CLES or COMES without substrates were gently transferred onto the corneal surface with microforceps and then expanded on the bare corneal stroma with a surgical sponge or forceps. Substrate-free cell sheets were used in this experiment to avoid potential immune response related to the use of human amniotic membranes. Cell sheets were allowed to attach for 5 minutes without sutures. After surgery, all rabbits were fitted with a bandage contact lens and 0.3% ofloxacin ointment (Tarivid ointment; Santen Pharmaceutical Co.) and 0.1% betamethasone eyedrops (Sanbetason; Santen Pharmaceutical Co.) were applied twice daily. Animals were examined for epithelial integrity and any consequences related to epithelial sheet transplantation. Corneas were periodically photographed with and without fluorescein dye staining. Animals were killed at 5 weeks after sheet transplantation, and corneas were subjected to histologic and immunohistochemical examination.

In Vivo Measurement of Barrier Function Using Fluorophotometry
Barrier function in rabbit corneas with cultivated limbal epithelial transplantation (CLET) and COMET was assessed by fluorophotometry (Anterior Fluorometer FL-500; Kowa, Tokyo, Japan). We measured fluorescein concentration in the 0.3 × 0.5-mm central corneal stroma using a method previously reported. Briefly, background fluorescence intensity was measured in rabbits after anesthesia. Then, 3 μL of 0.5% sodium fluorescein was instilled into the rabbit eyes. Eyes were gently washed with sterile saline solution after 10 minutes, and fluorophotometric measurement was performed 20 minutes later. After subtraction of background fluorescence from the measured value, it was expressed as a concentration of fluorescein (ng/mL). Measurements were performed before and 2 weeks after preparation of the limbal deficiency model and 5 and 10 weeks after epithelial sheet transplantation.

Statistical Analysis
Statistical comparisons between two different conditions were performed with the nonpaired Student’s t-test (Excel; Microsoft, Redmond, WA). Correlation between TER and assay (LC-Biotin; Pierce) was assessed using software (CORREL; Excel; Microsoft).

RESULTS

Immunohistochemistry and Electron Microscopy
Both normal human corneas and CLES showed positive staining for K3, especially in the epithelial cells, but were negative for K13. Normal oral mucosa and COMES showed positive staining for K3. Both were strongly positive for K13 throughout the epithelial layers (Fig. 1).

Both normal human corneas and oral mucosa were positive for occludin and ZO-1; however, the distribution of these TJ-related proteins was remarkably different. In the cornea, ZO-1 and occludin were located exclusively at the apical surface of the superficial epithelium, but in the oral mucosa they were abundantly distributed in the intercellular junctions throughout all the epithelial layers (Fig. 1). CLES showed a staining pattern similar to that of normal cornea: both occludin and ZO-1 were located at the apical epithelium. Distribution of TJ-related proteins in COMES was considerably different from that in normal oral mucosa. They were seen only in the apical epithelial cells in COMES, similar to distribution in normal cornea and CLES. These immunohistochemical findings were similar in rabbits (data not shown). Transmission electron microscopy revealed that both CLES and COMES demonstrated TJ-like structures in the apical portion of the superficial epithelium (Fig. 2).

Epithelial Barrier Function by LC-Biotin Assay
First, assay (LC-Biotin; Pierce) was applied to normal cornea with localized epithelial damage to confirm the validity of the assay. We found that it did not penetrate intact corneal epithelium and remained on the apical surface of the epithelium. However, the dye was seen in the corneal stroma in the area where the epithelium was injured (Fig. 3A).

Assay (LC-Biotin; Pierce) remained in the superficial epithelial layer of normal oral mucosa, and no dye penetration into the substantia propria was noted (Fig. 3B). Although no penetration of was observed in human CLES (Fig. 3D), it was seen in the intercellular spaces and in the underlying AM in rabbit CLES (Fig. 3C). Barrier function against the assay was well maintained in both human and rabbit COMES (Figs. 3C, 3D). Differences in grading scores between rabbit CLES and COMES were statistically significant (Fig. 3E; P < 0.001).

Epithelial Barrier Function Using TER
The TER values obtained in various epithelial sheets are shown in Figure 4. Regardless of culture conditions used (explant or cell suspension; presence or absence of substrates), rabbit CLES demonstrated low TER values (decreased barrier function). In contrast, rabbit COMES showed significantly larger TER values than CLES, indicating that barrier function was better in the former (Fig. 4A; P < 0.001). In human epithelial sheets, both CLES and COMES demonstrated intermediate TER values under various cultivation conditions. No significant differences were noted between explant and cell suspension or between presence and absence of AM (Fig. 4B).

Correlations between assay (LC-Biotin; Pierce) grading scores and TER values in the same rabbit and human samples are shown in Figures 4C and 4D, respectively. The two values showed good correlation in both rabbit (r = −0.87) and human (r = −0.75).

Epithelial Sheet Transplantation to Rabbits
Two weeks after superficial keratectomy including the limbus, rabbit corneas were covered with fibrovascular tissue. Some corneas showed persistent epithelial defects (Fig. 5A). After transplantation of cell sheets, all corneas showed regression of
opacity and vascularization. Control corneas with no epithelial transplantation were totally covered with fibrovascular tissue (data not shown). Some differences were observed in postoperative outcomes between eyes that had undergone CLET and COMET; whereas all corneas maintained clarity with epithelial integrity after CLET, some corneas showed an irregular epithelial surface associated with peripheral superficial neovascularization in eyes that had undergone COMET (Fig. 5B).

**Figure 1.** TJ-related proteins and differentiation markers in human CLES and COMES. Immunohistochemistry of K3 (a), K13 (b), occludin (c), and ZO-1 (d) in human cornea (A), human CLES (B), human COMES (C), and human oral mucosa (D). Nuclei of cells were stained with DAPI. Scale bars, 50 μm.

**Figure 2.** Transmission electron micrographs of human CLES and COMES. Both CLES (A) and COMES (B) showed TJ-like structure formation in apical cells (arrows). Scale bar, 1 μm.
In Vivo Fluorophotometry

In vivo fluorophotometry revealed that barrier function in normal rabbit cornea was comparable to that in human. Two weeks after superficial keratectomy, values increased by approximately 12-fold, indicating that barrier function was markedly impaired ($P < 0.00002$). Significant recovery of barrier

**Figure 3.** Epithelial barrier function by biotin assay. Surface of rabbit tissue (A, cornea; B, oral mucosa), rabbit epithelial sheets (C), and human epithelial sheets (D) exposed to biotin assay and stained for immunofluorescence assay. Nuclei of cells were stained with DAPI. Scale bars: 100 $\mu$m (A, B); 50 $\mu$m (D). *$P < 0.001.$
function was noticed after CLET \((P = 0.002)\), and values were similar to those before surgery. However, values remained considerably high after COMET (Fig. 5C). Differences in fluorometric values between CLES and COMES at 5 weeks after surgery were statistically significant \((P = 0.025)\). Values similar to those at 5 weeks after surgery were also observed at 10 weeks after epithelial transplantation (data not shown).

**Histologic and Immunohistochemical Examination after Epithelial Sheet Transplantation**

Five weeks after CLET, the cornea was covered with smooth and regularly stratified epithelia. They were well attached to the underlying stroma, and infiltration of the superficial stroma by inflammatory cells was mild (Fig. 6A). Immunohistochemistry revealed that the epithelium was positive for K3 but stained weakly in the superficial layer for K4. Both occludin and ZO-1 were positive in the apical surface of the epithelium (Fig. 6D).

In eyes that had undergone COMET, epithelia showed some irregularity in thickness. Although four to five layers of epithelial cells covered the corneal surface in some areas, there were five to 10 layers in other areas (Fig. 6C). Inflammatory response in the corneal stroma was mild. Immunohistochemistry revealed weak staining for K3 but strong staining for K4. Both occludin and ZO-1 showed positive staining in the superficial layer where four to five layers of epithelium had formed (Fig. 6E) but showed diffuse staining patterns where the epithelium had thickened (Fig. 6F). Although occludin was positive throughout all the epithelial layers, including the superficial cells of the thickened epithelium, ZO-1 was positive in the wing layer but not in the superficial layer of the epithelium (Figs. 6E, 6F).

**DISCUSSION**

In the present study, we found that both occludin and ZO-1 were expressed on the apical surfaces of CLES and COMES in both rabbit and human, and these results are in good accordance with those of previous reports.\(^2\) It is intriguing that the distribution of occludin and ZO-1 was considerably different between normal oral mucosa and COMES. Whereas they were diffusely distributed intercellularly throughout the epithelial layers in the oral mucosa, they were localized predominantly in the superficial epithelium in COMES. Distribution of TJ-related proteins was reported to be different among different stratified epithelia, and diffuse distribution of TJ-related proteins in the lower strata of the oral tissues such as the gingiva was demonstrated.\(^1,30\) This suggests that expression of TJ-related proteins is not directly related to barrier function. Sugrue and Zieske\(^31\) reported positive ZO-1 staining in rabbit cornea not only in the apical cells but also in the basal epithelial cells. Immunoelectron microscopy revealed that mid-epithelial accumulations of ZO-1 were not TJs but rather a form of adherens junction. Expression of ZO-1 in the mid-epithelial level of the cornea was correlated neither with the presence of TJs nor with established barrier function in their study.

We assessed epithelial barrier function using three methods: assay (LC-Biotin; Pierce), TER measurement, and in vivo fluorophotometry. In vitro assessment of barrier function using assay (LC-Biotin; Pierce) and TER showed excellent correlation.
Neither method, TER measurement in particular, is invasive, making both suitable for preoperative evaluation of cultivated epithelial sheets. Our results indicate that the barrier function of CLES and COMES was influenced by various factors, such as cell source and culture condition. We noticed that TER values were significantly larger (better barrier function) in COMES than in CLES in rabbit, but there were no such differences in human (Figs. 4A, 4B). We believe that the culture conditions for CLES used in the present study may not be optimal for rabbits because they were designed for clinical application. It should be noted, however, that assay (LC-Biotin; Pierce) has a relatively small molecular weight (556.59), similar to that of fluorescein sodium (376.27). Therefore, decreased values in these measurements do not always lead to impaired barrier function against molecules of larger weight.

We also observed marked differences in barrier function between in vitro and in vivo conditions. Although rabbit COMES showed excellent TER values in vitro, recovery of barrier function after COMET in rabbit limbal deficiency models showed no improvement over that with CLET. The decreased barrier function after COMET seen in this study is in good accordance with that of our previous report. One possible explanation regarding the decreased barrier function in COMES is that its expression in mucin components is different from that in normal ocular surface epithelia. Although COMES has been reported to express membrane-associated mucins similar to those in CLES, the former does not express secreted mucins. Given that the mucin layer contributes to a barrier to fluorescein dye in ocular surface epithelia, alteration in mucin may result in decreased barrier function. Kimura et al. reported that TNF-α induced a decrease in expression of ZO-1, but not in occludin, in corneal epithelial cell lines. Decreases in ZO-1 were correlated with decreased barrier function measured by TER in their study. Similar changes in barrier function in other types of epithelia have also been reported. Furthermore, other environmental changes, such as hypoxia, were reported to interfere with corneal epithelial barrier function, and decreases in function can be reversed by keratinocyte growth factor. We observed that the expression of ZO-1, but not occludin, was decreased in the superficial cells of the thickened epithelium after COMET (Figs. 6E, 6F). It is possible that this downregulation of ZO-1 is related to decreased barrier function after COMET. Although the mechanism of this alter-
It should be noted, however, that our clinical experience, as well as that of others, indicates that episodes of postoperative infection in patients who have undergone COMET are rare compared with those in patients who have undergone CLET using allogeneic sources (YS, unpublished data, 2009). It seems that susceptibility to infection is more affected by other factors, such as use of immunosuppressants.

In summary, we found that barrier function in cultivated epithelial sheets was influenced by various factors, such as cell source and cultivation method. Although TJ-related proteins such as occludin and ZO-1 were expressed in superficial epithelium in CLES and COMES in vitro, in vivo barrier function after COMET showed a decrease in comparison with that after CLES. Whether these observations are clinically relevant will require confirmation through further study.

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